POTYVIRUS RESISTANCE DERIVED FROM ANTISENSE RNA AND NATIVE OR CHIMERIC COAT PROTEIN GENES

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Abstract

An antisense (AS) RNA construct consisting of the C-terminal portion of the coat protein (CP) gene and complete 3' non-coding sequence of bean yellow mosaic virus (BYMV), and driven by the cauliflower mosaic virus (CaMV) 35S promoter, was used to obtain transgenic *Nicotiana benthamiana* plants by *Agrobacterium*-mediated transformation. Other plants were transformed with constructs designed to express the BYMV CP gene or chimeric CP genes. The original transformants from each construct were allowed to self. R1 plants carrying the introduced gene were selected on the basis of polymerase chain reaction (PCR) and/or ELISA (for CP-expressing plants) with monoclonal antibodies. Homozygous R2 plants expressing AS RNA displayed a range of resistance, from minimal to apparent immunity from infection by BYMV; no resistance was observed to other potyviruses. Plants expressing native BYMV CP also showed a range of resistance, with a minimal degree of resistance to other potyviruses. Both AS and CP plants displayed two types of resistance; to initial infection, and/or to replication or movement. Chimeric CPs, with the N-terminal domains of BYMV fused to the C-terminal domains of pepper mottle potyvirus or zucchini yellow mosaic potyvirus, differed in their response to challenge with several potyviruses. At least one transformant of each chimeric CP showed milder symptoms than non-transgenic controls when inoculated with BYMV, and some resistance to potato virus Y. Deleted constructs are being prepared with the aims of separating the two types of resistance, determining the mechanisms of resistance, and which domains confer viral specificity.

1. Introduction

Coat protein (CP)-mediated resistance has been shown to be effective in a number of plant systems, against various viruses (reviewed by Beachy et al., 1990). Differences have been observed between different virus groups in the extent and mechanisms of CP-mediated resistance (discussed in an earlier paper; Hammond & Kamo, 1992). It has been reported that the CPs of soybean mosaic virus (SMV; Stark & Beachy,

1989) and papaya ringspot virus (PRV: Ling et al., 1991) confer resistance to other potyviruses; in contrast Lindbo & Dougherty (1992b) reported that the CP of tobacco etch virus (TEV) did not protect against two other potyviruses. Lindbo & Dougherty (1992a) have also shown that deleted forms of the TEV CP were at least as effective as full-length TEV CP against TEV infection.

Antisense (AS) RNA-mediated resistance to potato virus X (Hemenway et al., 1988) and cucumber mosaic virus (CMV; Cuozzo et al., 1988) was found to be less effective than CP-mediated resistance, and Rezaian et al., (1988) found that only a single transformant of one construct out of three tested conferred any measurable resistance against CMV. In contrast, TEV and BYMV AS RNA conferred resistance equal or superior to that from CP (Lindbo and Dougherty, 1992a,b; Hammond and Kamo, 1992).

More than one mechanism appears to be involved in both CP- and AS-mediated resistance. Systemic spread of TMV in CP-expressing plants is reduced even when RNA overcomes the initial resistance to infection (Wisniewski et al., 1990), and similar distinctions have been reported between resistance to initial infection and symptom reduction in plants expressing potyvirus CPs (Ling et al., 1991, Lindbo and Dougherty, 1992a,b; Regner et al., 1992; Hammond and Kamo, 1992) or AS RNA (Hammond & Kamo, 1992). We are examining CP, chimeric and deleted CP, and AS constructs from BYMV as a source of practical resistance, and as a means of determining the multiple mechanisms involved. We chose to use *Nicotiana benthamiana* because this species is susceptible to a broad range of potyviruses, including BYMV.

2. Materials and Methods

2.1 Gene constructs, plant transformation and assay

The BYMV CP-1, CP-2 and AS constructs have been described; CP-1 has a methionine initiation codon and 13 additional N-terminal amino acids derived from the nopaline synthase gene, while CP-2 has a methionine initiation codon added to the BYMV CP coding sequence (Hammond & Kamo, 1992); for the deleted BYMV constructs, C-terminal deletions were fused to the oligonucleotide-derived leader and initiation codon of construct CP-2; the deleted constructs were also expressed in *Escherichia coli* as fusions to the lacZ alpha peptide (Hammond & Hammond, 1989; Hammond et al., 1990). Chimeric constructs were made by in-frame fusions of the N-terminal coding sequences of CP-2 with C-terminal sequences of pepper mottle virus (PeMV; Dougherty et al., 1985) or zucchini yellow mosaic virus (ZYMV; Grumet & Fang, 1990) at common restriction sites; these constructs are termed BY/Pe and BY/ZY respectively.

Full details of the constructs, plant transformation and screening will be presented elsewhere. Briefly, the viral sequences were separately transferred to the binary vector pGA643 (An et al., 1988), transferred into *Agrobacterium tumefaciens* strain 5922, and used to transform *N. benthamiana*; regenerated plantlets, R1 and R2 populations were screened for the presence of the desired gene by polymerase chain reaction (PCR) essentially according to McGarvey and Kaper (1991), and (for CP plants) by ELISA with monoclonal antibodies PTY 1 and PTY 43 (Jordan and Hammond, 1991). Transformed plants were designated "CP-1","CP-2","AS","Pe" and "ZY". R2 populations that were apparently homozygous were used for all virus challenge experiments.

2.2 Virus purification, inoculation and assay

Turnip mosaic virus (TuMV) isolate Linc-2 (Hammond and Chastagner, 1988), BYMV Ideal A, BYMV-GDD, PeMV NC 165 and potato virus Y (PVY-3) were purifed as described (Hammond & Lawson, 1988). Purified virus was diluted to 100, 20 or 2 ug/ml, and 50 ul used to inoculate each of three leaves of four to eight plants for each transgenic population examined, and for the non-transgenic controls; two to five additional plants served as uninoculated controls. RNA of BYMV-GDD (Hammond & Lawson, 1988) at 2 or 20 ug/ml was inoculated similarly. Plants were observed daily for a period of four weeks after inoculation; ELISA assays for detection of transgene CP and virus replication were as described (Hammond & Kamo, 1992). Bioassay was performed by inoculation to *N. benthamiana* from selected plants.

3. Results

3.1. Transformation and gene detection

A minimum of five individual transformants of each construct have been taken to putative homozygosity at the R2 level(Hammond & Kamo, 1992). For each of the CP constructs a range of expression levels has been observed, from c.0.1-10% of the CP found in an active BYMV infection. The level of expressed CP was found to vary with leaf position and plant age; in general higher levels of CP were detected in younger leaves and younger plants (data not shown).

3.2. Challenge inoculation with BYMV

In most cases the transgenic plants became infected, initially developing symptoms indistinguishable from the non-transgenic controls. As the infection progressed, however, plants of at least one transformant of each construct (AS, CP-1, CP-2, Pe and ZY) showed remission of symptoms; for some transformants later-produced upper

leaves were free of symptoms. Symptomless upper leaves were free of virus as determined by ELISA and bioassay, and leaves with reduced symptoms generally had reduced virus titer compared to leaves in similar positions on plants with typical symptoms (Table 1). Some plants of some CP-1 and CP-2 transformants escaped infection with BYMV in experiments where all of the non-transgenic controls, and all plants of other transformants, became infected. All plants of one AS transformant, E6 AS 140, escaped infection from BYMV at all virus concentrations up to and including 100 ug/ml.No differences were observed between inoculation with virus or purified RNA; plants were equally resistant to either form of inoculum.

3.3. Challenge inoculation with other potyviruses

In experiments where CP-1, CP-2 and AS plants were challenged with BYMV, PeMV or TuMV at 100 ug/ml, no resistance was observed to PeMV or TuMV. In further experiments, CP-1, Pe and ZY plants were inoculated with BYMV, PVY and TuMV at 2 and 20 ug/ml, and two Pe transformants recovered from symptoms in later growth; no virus could be detected in these leaves (Table 1). All three Pe transformants had some resistance to infection with TuMV; some plants remained un-infected, and two plants of one transformant (Pe 30) showed at least a seven day delay in symptom production (data not shown).

3.4. Expression of deleted CP constructs in bacteria

A series of C-terminal deletions of the BYMV CP gene were expressed in *E. coli* and found to produce stable, ELISA-detectable protein (Hammond et al., 1990). At present two of these constructs have been used to transform leaf disks of *N. benthamiana*, but plants have not yet been regenerated.

4. Discussion

Each of the CP and AS constructs conferred resistance to BYMV in *N. benthamiana*. The level of protection differed between individual transformants of the same construct, and was not correlated to the absolute amounts of CP produced in the transformant. This is consistent with previous reports of potyvirus CP-mediated protection (Lawson et al., 1990). Two separable mechanisms of resistance were observed in both CP and AS plants: resistance to initial infection; and resistance to replication and/or movement in plants that became infected. Both mechanisms were active in some transformants. The most effective resistance was observed in an AS transformant that resisted inoculation with up to 100 ug/ml of BYMV, and was apparently immune to infection (Hammond & Kamo, 1992). RNA was not able to overcome the resistance conferred by either AS or CP constructs, whereas

TMV RNA overcomes the resistance to virus (Wisniewski et al., 1990). This provides further evidence that the mechanisms of CP-mediated resistance are not equivalent for all virus groups.

The additional 14 amino acids in CP-1 did not seem to adversely affect resistance to BYMV, although Regner et al. (1992) have reported that additional plum pox virus (PPV) amino acids from the upstream replicase gene apparently inhibited protection to PPV. Lindbo & Dougherty (1992a) have reported that N- and C-terminal deleted TEV CPs conferred equivalent or superior protection to full-length CP, so it appears that the terminal domains are not required for, and additional amino acids may not interfere with, protection.

Although some resistance to PVY (recovery from symptom expression, with no virus detectable in such leaves) and TuMV (resistance to infection and delay in symptom appearance) was observed in the Pe plants, broad spectrum resistance as reported with other potyvirus CPs (Stark & Beachy, 1989: Ling et al., 1990) was not observed. Lindbo and Dougherty (1992b) did not observe any protection against heterologous potyviruses from the TEV CP, and we did not see significant protection against heterologous viruses with the BYMV CP-1 and CP-2 constructs. The chimera we have described as Pe should perhaps be described as PY, as Vance et al. (1992) have shown that the virus called PeMV by Dougherty et al. (1985) is distinct from a California isolate of PeMV, and should probably be regarded as a strain of PVY. The resistance to PVY observed in the current experiments may be attributable to the presence of the C-terminal, PeMV (PVY ?) portion of the chimera; however whether this is the case, and whether amino acid sequence or the 3' non-coding sequence is responsible cannot be determined on the basis of these experiments. Additional viruses will be used to challenge plants expressing the BYMV CP, chimeric and deleted CP constructs to determine the contribution of each domain to each of the observed mechanisms.

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Table 1 - Virus and CP levels detected by ELISA in control and inoculated plants, relative to leaf position and symptoms.

Plant Inoculum	Lower	Middle	Upper	Symptoms
NT				
NI control	0.01	0.01	0.01	NS
2 ug/ml BYMV	0.31	0.15	0.31	mm
CP-1 79				
NI control	0.01	0.02	0.04	NS
2 ug/ml BYMV	0.09	0.02	0.01	mm/IC/RAT
ZY 7				
NI control	0.01	0.01	0.01	NS
2 ug/ml BYMV	0.01	0.19	0.40	vmm
2 ug/ml BYMV	0.33	0.09	0.48	mm
ZY 43				
NI control	0.01	0.02	0.05	NS
2 ug/ml BYMV	0.06	0.30	0.02	mm/IC/RAT
Pe 9				
NI control	0.02	0.02	0.05	NS
2 ug/ml BYMV	0.02	0.02	0.04	vmm
20ug/ml PVY **	0.04	0.18	0.02	mm/GVB/RAT
Pe 30				
NI control	0.06	0.09	0.15	NS
20ug/ml BYMV	0.09	0.37	0.02	mm/IC/RAT
20ug/ml PVY **	0.07	0.33	0.02	mm/GVB/RAT

Leaf samples harvested 30 days after challenge.

NT = non-transgenic; NI = not inoculated; NS = no symptoms; mm = mild mosaic; vmm = very mild mosaic; IC = interveinal chlorosis; RAT = recovery at top (symptomless upper leaves); GVB = green vein banding.

** These transformants were the only ones in which recovery from PVY symptoms was observed.

SURVEY ON VIRUS DISEASES OF BULBOUS FLOWERS IN HUNGARY

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Abstract

More than 400 tulip plants belonging to 95 cultivars and 6 species showing typical symptoms of viral infections were examined. In the samples tulip breaking virus (TBV), tobacco rattle virus (TRV), tobacco necrosis virus (TNV) and cucumber mosaic virus (CMV) were identified. These viruses were frequently found in complex infections.

Fresia leaves, corms and flowers with virus symptoms originated from growers and flower markets were surveyed, too. In 140 samples freesia mosaic virus (FMV), bean yellow mosaic virus (ByMV), cucumber mosaic virus (CMV) and tobacco rattle virus (TRV) were observed. FMV in double infection with ByMV is common. This is the first report of FMV, ByMV, CMV and TRV in freesia in Hungary.

1. Introduction

The economic importance of bulbous flowers has increased in Hungary. Their growing is more and more wide-spread among farmers. The commerce of propagation material - - instead of the earlier centralized one - is free for anybody. Virus diseases of these ornamentals have been unexplored in Hungary. That is why I have given my attention to this field. My work starting from 1989 has comprised virus diseases of tulips and freesia.

The virus diseases of tulips (*Tulipa spp.*) are wide-spread all over the world. The most frequent ones are tulip breaking virus (TBV), tobacco rattle virus (TRV), cucumber mosaic virus (CMV), tobacco necrosis virus (TNV) and lily symptomless virus (LSV). Their infection and damage have been reported by several researchers (table 1). Occasionally the following infrequent viruses can also be met with: arabis mosaic virus (AMV), tobacco ringspot virus (TRSV), tobacco mosaic virus (TMV), tomato bushy stunt virus (TBSV), tulip chlorotic blotch virus (TCBV) and tulip virus X (TVX) (table 2).

Though the number of viruses infecting freesia (*Freesia refracta hybrida*) is smaller, their significance and incidence are similar to tulip viruses. Bean yellow mosaic virus (ByMV) (Derks et al., 1987; Pleše, 1985),freesia mosaic virus (FMV) (Foxe et al., 1985; Dorst, 1973; Bellardi et al., 1989, cucumber mosaic virus (CMV) (Bellardi et al., 1989) have been found to infect naturally this plant. Leaf necrosis (LN), severe leaf necrosis (SLN) have been described as virus--like disorders (Dorst, 1973). Foxe et al. (1985) identified a virus belongs to the closterovirus group beside freesia mosaic virus. Freesia streak virus (FSV) was reported by Casper et al. (1971) beside the viruses mentioned above.

The aims of my present paper are to explore and identify the viruses that occur in Hungary, wich have probably been introduced from abroad due to the intensive importing activity, as well as to point out the state of being infected with viruses on the basis of symptoms.

2. Material and Methods

2.1. Origin of plant samples

In March-April, 1989-91 I collected plants showing viral symptoms from field grown tulip stands, from 3 farms, 2 collections. I made the gathering from 2-leaf stage up to the senescence of leaves after blooming. I stored the more than 400 samples of leaves and flowers of 95 cultivars from Tulipa gesnerana, T. fosterana, T. greigii, T. kaufmanniana, T. praestans, T. tarda at + 4 °C until examinations.

The freesia samples (140 pcs) were gathered from 3 farms (from plants grown under glass houses), from imported corms showing necrotic symptoms as well as from cut flowers sold in the market. I made the collecting in 1990-92. Each sample showed symptoms of virus infection.

2.2. Antisera

Antisera to tulip breaking virus (TBV-RE), tobacco necrosis virus (TNV-QM), tobacco rattle virus (TRV_{TF}, TRV_{TJ}), lily symptomless virus (LSV), bean yellow mosaic virus (ByMV-PZ) and freesia mosaic virus (FMV-SC) were prepared and provided by A.F.L.M. Derks (Bulb Research Center, Lisse) while to cucumber mosaic virus (CMV-N) was provided by Institute für Phytopathologie, Aschersleben. All antisera were stored at -18 °C.

2.3. DAS-ELISA

I homogenized the plant samples of tulips (leaves, flowers and bulbs by PBS-Tween puffer (pH 7.4) diluted sixfold. In case of the freesia samples I used the homogenizing puffer suggested by the Bulb Research Center (Lisse), (8 g NaCl, 1 g KH_2PO_4, 14.5 $Na_2HPO_4 \cdot 12\ H_2O$, 0.2 g KCl, 0.5 ml Tween 20 in 1000 ml distilled water; pH 7.4)[2] also with sixfold dilution. The diluted sample extracts settled at +4 °C for 1-2 days to direct double antibody sandwich (DAS) ELISA examinations.

The ELISA method was applied according to Clark and Adams (1977), with modifications: omitting PVP from extraction puffer and adding 0.4 % horse serum to conjugate buffers instead of BSA. Both healthy and known infected controll samples were given by Bulb Research Center. Plates were valued by visually and measured at 405 nm with Dynotech MR 250 spectrophotometer, too. Absorbance values above 0.150 were considered positive (infected) in case their values were at least the treble of the virus-free control.

2.4. Electron microscopy

Electron microscopic examinations from crude extracts of leaves and flowers (petals) were done following negative staining in 2 % solution of uranyl acetate. In some cases I applied immunosorbent electron microscopy (ISEM) technique, too (Hill, 1984), when virus concentrations were too low to permit detection.

3. Symptoms and Incidence of Viruses

3.1. Tulips

Each of the 417 tulip samples gathered from different places showed typical virus symptoms so by visual assasment I collected - in all probability - infected plants. To serological and electron microscopic examinations I used leaf- and flower samples.

In the major part of the samples I observed the typical symptoms of tulip breaking virus (TBV) . At an early phenological stage, at two-leaf stage, in certain cultivars redding purple, red colours frequently on leaf edges and light and dark green mosaic develop on leaves. Later green and yellowgreen areas, islands streaks and bands of irregular shape and size appear on the whole surface of the leaf or only on the tip.

Due to the serious TBV infection the shape of the flower bud frequently changes, becomes slender. The colour-breaking of the flower petal is already striking and distinctly visible at this stage. Flower petals grow often narrow, their shape becomes irregular, their edges get wavy. The colour-breaking of petals appear in different ways. The stigma may also show colour change many times. After blooming in certain cultivars silvergrey oval spots and rings develop while earlier no other symptoms could be seen. I found TBV in 59 % of the examined 417 samples.

At a significant part of the samples (25 %) I observed the presence of tobacco rattle virus (TRV). It generally occured together with TBV or/and TNV so the developing symptoms were not characteristic. I found single infection with TRV in one case only and on the leaves of this plant grey-green lozengeshape and elongated spots developed.

Tobacco necrosis virus (TNV) could be observed in almost 11 % of the samples, but only in complex infection. In some cases chlorotic and necrotic streaks and elliptical necrotic spots appeared on the leaves. At serious infection necrosis may develop on the flowers causing their deformation.

Cucumber mosaic virus (CMV) could be met with only in four samples: in one of them could be found single infection. On the leaves of this plant brown necrotic streaks developed along the vein and at the edges, as well as on the petals of flower bud. The leaves were slightly deformed and the plant showed growth reduction. I have not managed to identify lily symptomless virus (LSV) in any of the examined tulip samples.

3.2. Freesias

On the leaves and flower stalk of virus infected freesia collected from 3-leaf stage to blooming, showed chlorosis, necrosis, later colour-breaking and flower deformation could be observed. In the samples of 75 plants grown under glass, gathered from different counties, freesia mosaic virus (FMV) 32 %, bean yellow mosaic virus (ByMV) 12 %, cucumber mosaic virus (CMV) 4 % occured in single infections. These three viruses were detected in complex infections in 34 % of samples.

I grew under glass imported freesia corms covered by necrotic spots to different extents. From those 33 corms only 19 sprouted while the others decayed being unviable. FMV was found in 6 plants, FMV with ByMV in 13 ones while ByMV could not be observed in single infection. CMV could not be found in the samples. From the same corm stock I examined 14 corms 6 month later by ELISA and the following viruses were detected generally in complex infection: FMV - 12 pcs, ByMV - 6 pcs and tobacco rattle virus (TRV) - 5 pcs. CMV was not found.

I also collected freesia flowers from market cultivated under glass, 18 species from 18 different places of origin. All of them showed the typical colourbreaking symptoms. The changes of the flower colour were due to the complex infection with FMV (17), ByMV (16) and TRV (3). CMV could not be observed in the samples.

4. Discussion

4.1. Virus diseases of tulips

In the gathered and examined tulip species and cultivars I identified the presence of TBV, TRV, TNV and CMV. Their frequency corresponds with the order of the list. LSV could not be detected in any of the samples. Though plants with symptoms of virus infection were collected only in 8 samples I could not detect the examined viruses. It may be supposed that other virus(es) also appear(s). Samples of leaves and flowers are suitable for serological virus diagnosis. On the basis of the symptoms i.e. the

colour-breaking of the flowers the infection with TBV can be detected - to all probability - by visual examination. The symptoms of the other viruses that I examined and identified are unspecific and similar. Complex virus infections cover up and change specific symptoms, idistinguish one from others visually is unreliable.

4.2. Virus diseases of freesias

From the viruses infecting freesia I observed the presence of FMV, ByMV, CMV and TRV. In the majority of the collected samples I identified complex forms of infections; FMV together with ByMV are general. Nevertheless, in many cases I detected the presence of three viruses. The symptoms caused by the examined viruses on the leaves and flowers are nearly the same, on the basis of them virus-identification is impossible.

The virusinfected tulip and freesia plants collected from stands occured dispersedly: they formed neither lines nor spots in growing field, wich would have indicated infection spreading. These facts prove the origin of virusinfection from propagation material.

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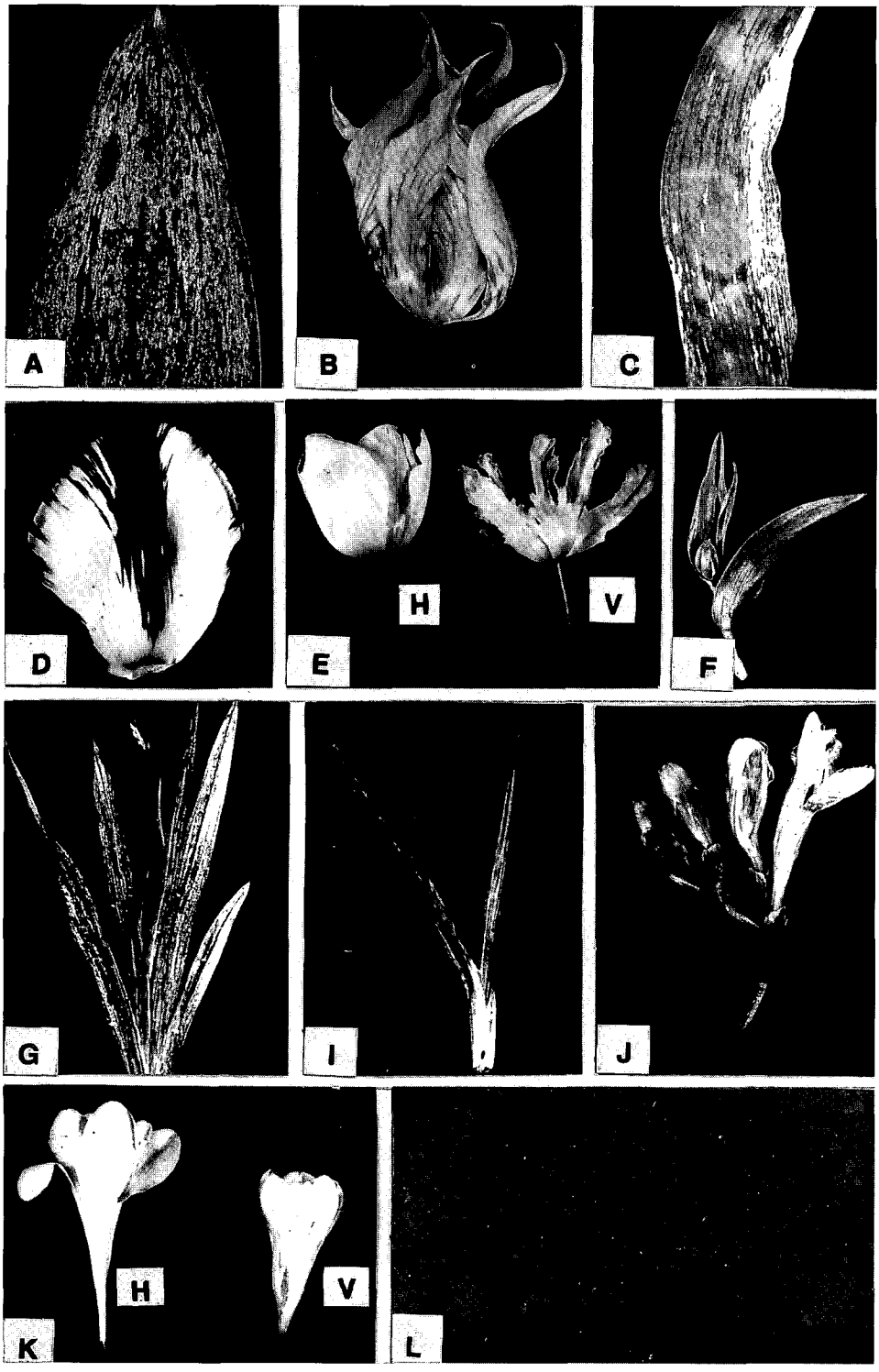
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Figure 1 - A. Grey and silvery ringspots and streaks on tulip leaf caused by TBV. B. Colour breaking and distortion of tulip flower infected with TBV. C. Chlorotic and necrotic spots and lines on TNV infected tulip leaf. D. White patterns in dark purple colour petal indicate TBV infection. E. Infection of TBV causes narrow petals and abnormal shaped flower. H: healthy, V: virus infected. F. CMV-infected tulip shows growth reduction, necrosis and deformation. G. Leaves of freesia showing chlorotic and necrotic streaks, spots caused by FMV. I. Freesia leaves infected with ByMV with yellow line patterns and rings. J. Deformation and colour breaking of freesia induced by FMV and ByMV. K. Virus infected flower (V) is smaller than heatly (H) and shows colour breaking. L. Particles of ByMV from freesia flower using ISEM technique. x42 000. Bar = 500 nm.

Table 1 - Important viruses isolated from tulips.

Name	Group	Particle dimension (nm)	Transmission	References
Tulip breaking virus (TBV)	POTYVIRUS	750	Aphids, Mechanical	Asjes (1982), Asjes (manuscript), Nahata et al.(1988)
Tobacco rattle virus (TRV)	TOBRAVIRUS	90x20-25 190x20-25	Nematodes	Matsunami et al. (1971), Whitlook (1983)
Cucumber mosaic virus (CMV)	CUCMOVIRUS	30	Aphids, Mechanical	Mowat (1970), Nahata et al.(1988)
Tobacco necrosis virus (TNV)	-	28,16	Fungal	Gáborjányi (1969), Lange (1977), Mowat (1970), Nahata et al.(1988)
Lily symptomless virus (LSV)	CARLAVIRUS	650	Aphids	Derks et al.(1975)

Table 2 - Tulip viruses with occasional occurrence.

Name	Group	Particle dimension (nm)	Transmission	References
Arabis mosaic virus (AMV)	NEPOVIRUS	28	Nematodes	Asjes (1976), Brunt et al.(1970)
Tobacco ringspot virus (TRSV)	NEPOVIRUS	28	Nematodes	Asjes (1970)
Tomato bushy stuntvirus (TBSV)	TOMBUSVIRUS	30	Mechanical	Mowat (1970)
Tobacco mosaic virus (TMV)	TOBAMOVIRUS	300	Soil	Mokra et al.(1973)
Tulip chlorotic blotch virus (TCBV)	POTYVIRUS	720	Aphids, Mechanical	Mowat(1985)
Tulip virus X (TVX)	POTYVIRUS	500	Mechanical	Mowat (1982)